

⁴⁹ Spiegelman, S., in *The Chemical Basis of Heredity*, ed. W. D. McElroy and Bentley Glass (Baltimore, Maryland: Johns Hopkins Press, 1957), pp. 232-267.

⁵⁰ Schachman, H. K., J. Adler, C. M. Radding, I. R. Lehman, and A. Kornberg, *J. Biol. Chem.*, **235**, 3242 (1960).

⁵¹ Berg, P., H. Fancher, and M. Chamberlin, in *The Synthesis of Mixed Polynucleotides Containing Ribo and Deoxynucleotides by Purified DNA Polymerase*, ed. H. J. Vogel, V. Bryson, and J. O. Lampen (New York: Academic Press, 1963).

⁵² Spiegelman, S., "Protein and nucleic acid synthesis in subcellular fraction of bacterial cells," in *Recent Progress in Microbiology*, VII International Cong. for Microbiology (Uppsala: Almqvist and Wiksells Boktryckeri AG.), pp. 81-103.

⁵³ Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

⁵⁴ Chamberlin, M., and P. Berg, *J. Mol. Biol.*, **8**, 297 (1964).

⁵⁵ Sinsheimer, R. L., and M. Lawrence, *J. Mol. Biol.*, **8**, 289 (1964).

⁵⁶ Bassel, A., M. Hayashi, and S. Spiegelman, these PROCEEDINGS, **52**, 796 (1964).

⁵⁷ Hayashi, M. N., M. Hayashi, and S. Spiegelman, *Biophys. J.*, **5**, 231 (1965).

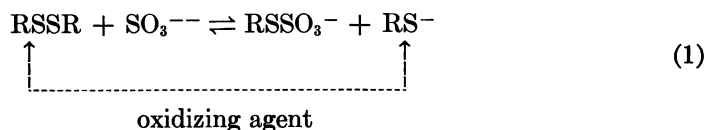
INSULIN SYNTHESIS BY RECOMBINATION OF A AND B CHAINS: A HIGHLY EFFICIENT METHOD*

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Impressive advancements in methods of synthesis and purification of peptides in the last 15 years have set the stage for the synthesis of polypeptide chains of length and complexity comparable to that of low-molecular-weight proteins.¹ On this premise, studies had been initiated in our laboratory directed toward the synthesis of insulin based on the assumption that air oxidation of a mixture of the sulfhydryl forms of chemically synthesized A and B chains should generate insulin.² Independent studies with natural insulin chains in two laboratories verified this assumption, even before the completion of our synthetic work. Dixon and Wardlaw³ and Du *et al.*⁴ reported the cleavage of insulin to its two chains by oxidative sulfitolysis, i.e., by reaction with sulfite in the presence of an oxidizing agent according to equation (1). Separation of the chains in the S-sulfonate form was followed by reconversion to their sulfhydryl form on treatment with a thiol. Finally, insulin was regenerated by air oxidation of a mixture of the sulfhydryl forms of the two chains.



The over-all process of cleavage and regeneration of insulin is illustrated in Figure 1. Subsequent to these reports we completed the synthesis and isolation in the S-sulfonate form of the A and B chains of sheep insulin,^{5, 6} and more recently, of human insulin.^{7, 8} Combination experiments between the sulfhydryl forms of the corresponding synthetic chains according to the scheme in Figure 1 led to the genera-

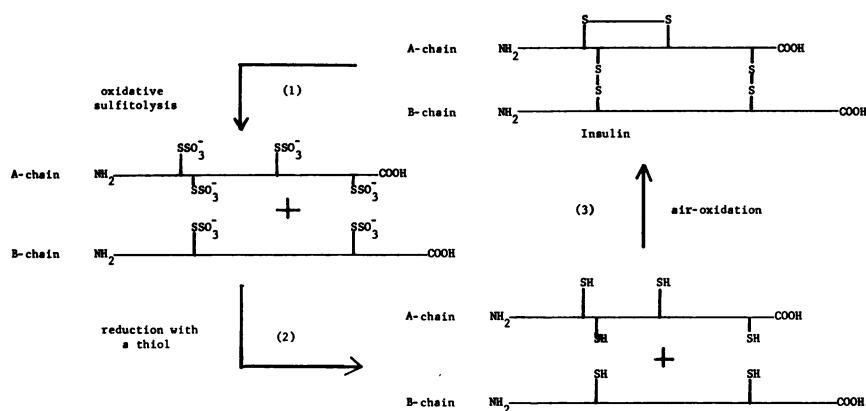


FIG. 1.—Cleavage and resynthesis of insulin from its separated chains: (1) sulfitolysis with $\text{Na}_2\text{SO}_3 + \text{Na}_2\text{S}_4\text{O}_6$; (2) conversion of the S-sulfonated chains to their sulfhydryl form by treatment with mercaptoethanol or thioglycolic acid; (3) air-oxidation of the reduced chains to form insulin.

tion of sheep and human insulin. In addition, combination experiments between these synthetic chains with the corresponding natural chains of bovine insulin by the same route produced the respective hybrid insulins. Initially, the over-all yield of insulin formed (calculated on the basis of the S-sulfonate of the B chain used) was approximately 2 per cent of theory when two synthetic chains were combined, and ranged between 4 and 8 per cent when either synthetic chain was combined with one prepared by oxidative sulfitolysis of natural insulin.⁹ The reported yields for insulin synthesis by combination of the sulfhydryl forms of natural A and B chains, prepared by oxidative sulfitolysis of insulin followed by reduction with mercaptoethanol, vary considerably. Dixon and Wardlaw³ obtained a yield of 1–2 per cent of theory, whereas Du *et al.*⁴ reported a yield of 5–10 per cent. More recently, Du *et al.*¹⁰ indicated a yield of close to 50 per cent of theory in insulin generation by recombination of natural A and B chains of bovine insulin. However, in this case it appears that the yield is not calculated on the basis of the amounts of the starting chains but on the basis of the specific activity of the final oxidation product. On the basis of the S-sulfonate of the B chain used, the yield of insulin produced by recombination of natural chains of bovine insulin obtained in our laboratory under the conditions followed by Du *et al.*¹⁰ was approximately 12–16 per cent of theory.⁹ In this paper we describe a method for combining the A and B chains to produce insulin in yields ranging from 60 to 80 per cent (based on the amount of S-sulfonate of the B chain used; uncorrected for water content of the chains which amounts to 5% per chain) when natural chains of bovine insulin are recombined. The improved yields of insulin were obtained by reaction of the S-sulfonated form of the B chain with severalfold excess of the sulfhydryl form of the A chain.

Experimental.—The water used in these experiments was deaerated by extensive boiling under nitrogen. All reagents used were of analytical grade. The natural S-sulfonated forms of A and B chains (ASSO_3^- and BSSO_3^- , respectively) were prepared by oxidative sulfitolysis of bovine insulin by a new procedure.^{9, 11}

Biological assays: Insulin determinations were carried out by the mouse convulsion method. Each unknown sample was assayed for 3–4 consecutive days. Each day a new insulin standard was used. Two concentrations of the standard and three concentrations of the unknown sample

were injected each day into five groups of 12–16 mice each. For the complete assay of a particular sample, a total of 180–200 mice were used. The reported assay values are the average figures of the 3- or 4-day assays. Highly purified crystalline zinc insulin (bovine) from Eli Lilly and Co., Indianapolis, with a specific activity of 25 IU, was used for the preparation of the reference standards.

Insulin synthesis by recombination of the sulfhydryl form of the B chain with a large excess of the sulfhydryl form of the A chain: A mixture of BSSO_3^- (10 mg) and ASSO_3^- (42 mg) was dissolved in water (9 ml) by adding a few drops of 1 *N* NaOH. The solution was subsequently adjusted to pH 5.0 with 1 *N* acetic acid (whereupon a homogeneous precipitate formed), cooled to 0°, and, after adding 1 *M* mercaptoethanol (1 ml) and deaerating with an aspirator, was heated in a boiling water bath for 6 min. The entire operation was carried out in a nitrogen atmosphere. The reaction mixture was then cooled, adjusted to pH 3.8 with 1 *N* acetic acid, and allowed to stand at 0°C for 10 min. The precipitated product was isolated by centrifugation (3000 rpm) and washed consecutively with 0.2 *M* acetate buffer (20 ml) pH 3.8 and 0.02 *M* acetate buffer (20 ml) pH 3.8. A suspension of this precipitate in water (8 ml) was solubilized by adjusting the pH to 10.6 with 1 *N* NaOH, mixed with 0.1 *M* glycine buffer (1 ml) pH 10.6, and stirred at 0° in contact with air for 24 hr. This solution which had a total volume of 10 ml was then assayed for insulin activity by the mouse convulsion method and found to possess 20 units of insulin per ml of solution. Since the theoretical amount of insulin generation, on the basis of the S-sulfonate of the B chain used, is 40 units per ml of solution (1.6 mg of insulin per ml), the over-all yield in insulin formation by this method is 50%.

Insulin synthesis by interaction of the sulfhydryl form of the A chain with the S-sulfonate form of the B chain: A solution of ASSO_3^- (20 mg) in water (5 ml) was adjusted to pH 5, mixed with 1 *M* mercaptoethanol (0.5–1 ml, 23- to 46-fold excess), deaerated (0°C), and heated in a boiling water bath for 4–8 min. The entire process was carried out in a nitrogen atmosphere. The reaction mixture was then cooled to 10–15°C and extracted four times with 40-ml portions of ethyl acetate. After the last extraction, the traces of ethyl acetate were removed by flushing the reaction vessel with nitrogen. The resulting jelly-like product was mixed with BSSO_3^- (5 mg) and water (7 ml). After adjusting the pH of the reaction mixture to 10.6 with 1 *N* NaOH, a clear solution was obtained. This solution was diluted with 0.1 *M* glycine buffer (0.8 ml) pH 10.6, stirred for 18–22 hr at 2°C, and then submitted to insulin assays. In 20 experiments the solution (total vol 10 ml) of the recombined product was shown to possess, by the mouse convulsion method, 12–16 insulin units per ml. Since the theoretical amount of insulin formed, based on the amount of BSSO_3^- used, is 20 units per ml (0.8 mg of insulin per ml), the recombination yield by this method ranges from 60 to 80%.

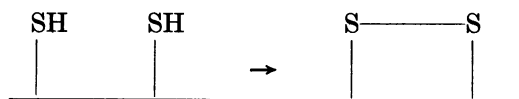
Insulin synthesis by interaction of the sulfhydryl form of the A chain with a large excess of the S-sulfonate form of the B chain: The recombination step was carried out as in the previous experiment except that only 10 mg of ASSO_3^- instead of 20 mg were converted to the sulfhydryl form and then allowed to react with 30 mg of BSSO_3^- . The solution (10 ml) of the oxidation product in three experiments was found to possess a total of 95–116 insulin units when assayed by the mouse convulsion method. Since the theoretical amount of insulin formed, calculated on the basis of ASSO_3^- used (10 mg), is 540 units, the recombination yield by this method ranges from 17 to 21% of theory.

Crystallization of insulin: The insulin formed by the recombination of natural bovine insulin chains was isolated and crystallized (Fig. 3) by the procedure described by Smith.¹² The crystalline material had an activity of 24 units per mg.

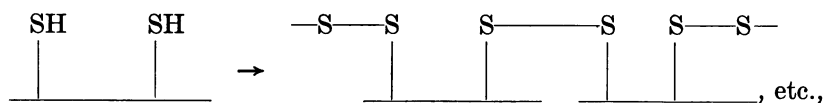
Results and Discussion.—The most efficient method for the resynthesis of insulin from its separated chains that has been reported thus far is the procedure described by Du *et al.*¹⁰ In this method a suspension of the S-sulfonates of the A and B chains, in a molar ratio of 1.5:1 in acetate buffer pH 5, is heated with a 25-fold excess of mercaptoethanol for 6 min at 100°C under nitrogen atmosphere. The sulfhydryl forms of the respective chains thus produced are precipitated at 0°C and at pH 3.8, and are then isolated by centrifugation and washed thoroughly with acetate buffer pH 3.8. Air oxidation of the reduced chains in glycine buffer at pH 10.6 and 3–5°C

leads to insulin formation. In a considerable number of experiments in our laboratory using this procedure, we obtained insulin in over-all yields ranging from 12 to 16 per cent of theory.⁹ The yield is calculated on the basis of the S-sulfonate of the B chain used and includes three distinctive steps: (1) the conversion of the S-sulfonates of the A and B chains to the sulfhydryl form; (2) the isolation of the reduced chains; and (3) the oxidation of the reduced chains to the final biologically active product. However, further studies in our laboratory on the recombination reaction revealed that during the precipitation of the reduced chains at pH 3.8 according to the method of Du *et al.*,¹⁰ extensive losses of the chains and particularly of the A chain occurred. Consequently, during the oxidation step, where the reduced chains are combined to form insulin, only a fraction of the original chains was present. The loss of chains is due mainly to their partial solubility in water even at pH 3.8, and to a lesser extent to unavoidable mechanical losses during the centrifugation and washing steps. In the present work, such loss was minimized by using a fivefold excess of A chain S-sulfonate and by using a high-speed centrifugation in the isolation of the sulfhydryl forms of the A and B chains. The yield of insulin in the experiment, in which both chains were in the sulfhydryl form, was thus brought up to 50 per cent of that theoretically possible with the amount of B chain used. This result also shows that, contrary to a recent report,¹³ oxidation of the reduced A chain prior to its reaction with the B chain is not necessary.

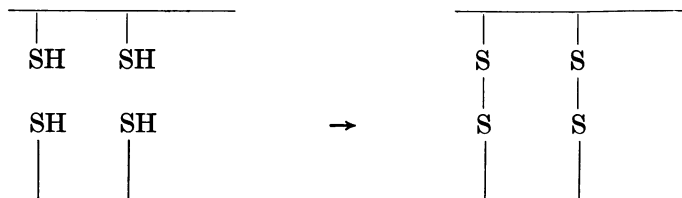
From the original work of du Vigneaud and co-workers¹⁴⁻¹⁶ on the synthesis of the neurohypophyseal hormones, it is clear that when the intermediate sulfhydryl nonapeptide is air-oxidized in dilute aqueous solution, intramolecular disulfide ring closure to form the active 20-membered cyclic octapeptide amide,



is favored over the linear polymerization,

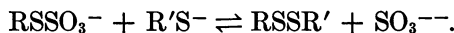


or the intermolecular disulfide bond formation,

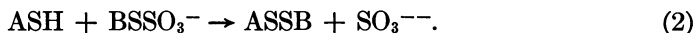


Comparable results were also obtained by Rydon¹⁷ in the oxidation of L-cysteinyl-tetraglycyl-L-cysteine. In this case too, the 20-membered cyclic monomer was formed in yields up to 90 per cent of theory, although a number of isomers are theoretically possible. It is therefore reasonable to expect that when the sulfhydryl forms of the A and B chains of insulin are subjected to air oxidation in dilute aqueous solution, disulfide ring closure to form the intrachain 20-membered cyclic

system of the A chain will precede the intermolecular disulfide bond formation between A and B chains. Obviously then, intermolecular disulfide bond formation, involving mainly sulfhydryl groups other than the ones participating in the intra-chain disulfide ring structure, between A chains, between B chains, and between A and B chains are probably the principal reactions that will compete during the oxidation of a mixture of reduced A and B chains. Of course even then the number of the possible isomers is indeed great. However, we have obtained insulin in yields up to 50 per cent of theory when reduced A and B chains were oxidized in dilute aqueous solution. This seems to imply that the necessary information for complementarity and covalent linking of the insulin chains to produce the protein is contained within the primary structure of the chains, and that of all the possible isomers that can be formed on air oxidation of the reduced chains, insulin is the predominant one. Subsequently, conditions that further reduce the number of isomers which might be produced by intermolecular disulfide bond formation between the same or different chains should increase substantially the yield of insulin generation. The procedure described in this report offers such conditions and results in high recombination yields. The basis of this method is found in equation (1). Since reaction 1 is reversible, it can be predicted that S-sulfonates will be converted by a thiol to the respective mixed disulfide as shown below:



Such conversions have been demonstrated¹⁸ for simple S-sulfonates, for S-sulfokera-teine, and even for insulin. Du *et al.*⁴ in their earlier work on the recombination of insulin chains have indeed caused the reduced A chain (ASH) to react with the S-sulfonate of the B chain (BSSO_3^-) and have generated insulin according to equation (2):



However, they did not pursue this route further.

We have now re-examined this reaction and found experimental conditions that

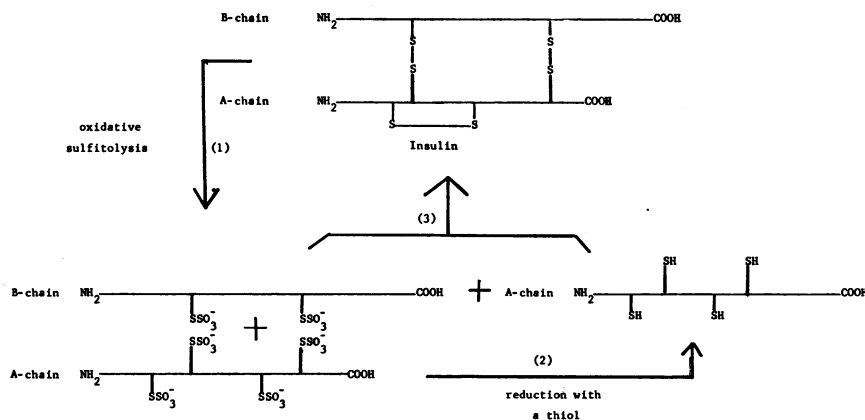


FIG. 2.—Cleavage and resynthesis of insulin by the improved method: (1) sulfitolysis with $\text{Na}_2\text{SO}_3 + \text{Na}_2\text{S}_4\text{O}_6$; (2) conversion of the S-sulfonate of A chain to the sulfhydryl form; (3) reaction of the sulfhydryl form of the A chain with the S-sulfonate of B chain to form insulin.

permit recombination yields ranging from 60 to 80 per cent (uncorrected for water content of the chains which amounts to 5% per chain), thus making it the most efficient method for recombining insulin chains to form the active protein. The over-all process of this improved method is illustrated in Figure 2. In view of the previous discussion, the results obtained by this recombination method are not surprising. The absence of free sulfhydryl groups in the B chain practically eliminates generation of isomers that might have been produced by intermolecular disulfide bond formation between B-chain molecules, and further diminishes the chances of any interference with the intramolecular disulfide ring closure in the A chain. However, the most important conclusion, from the high recombination yields obtained, is that the available sulfhydryl groups of the A chain react preferably with the appropriate S-sulfonate groups of the B chain to give the structure that corresponds to insulin. This strengthens the concept that the information needed for the folding and orientation of the A and B chains in a manner that permits their spontaneous combination to form insulin is embodied into the primary structure of the chains. It gives further support to the two-chain *in vivo* synthesis of insulin as suggested by Humbel.¹⁹ The conversion of the S-sulfonate of the A chain to its sulfhydryl form was carried out with mercaptoethanol at 100°C under nitrogen atmosphere. Contrary to published reports,¹⁰ the amount of mercaptoethanol used and the time of reaction, as judged from the yield obtained upon recombination with the B chain, is not critical. A 46-fold excess of mercaptoethanol

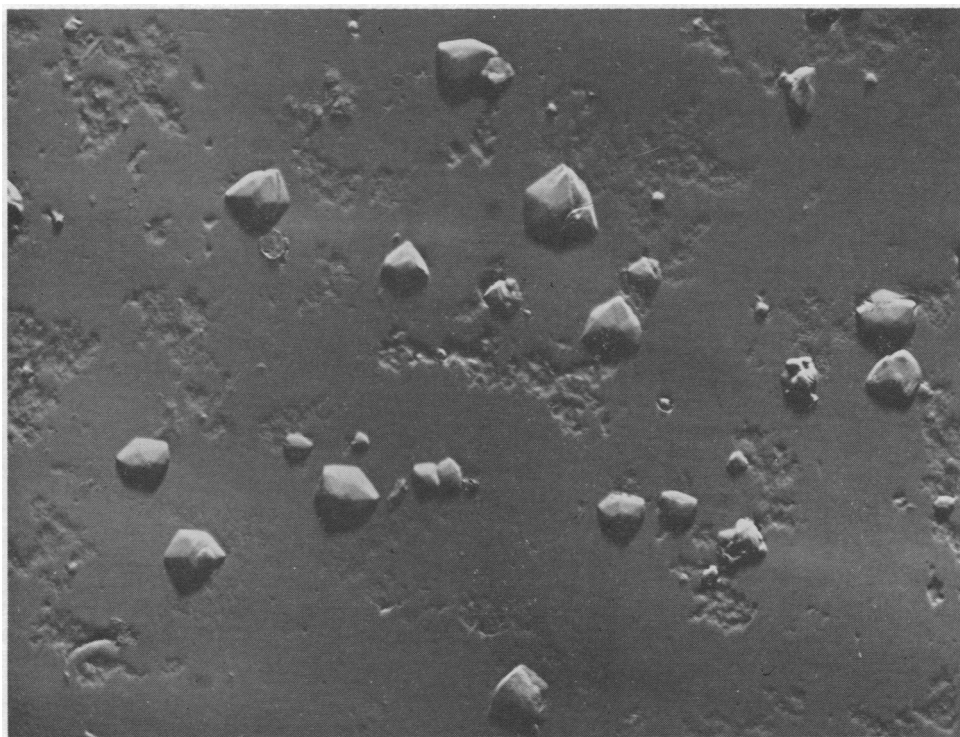


FIG. 3.—Crystalline insulin isolated from the recombination mixture of natural A and B chains of bovine insulin.

affords a sulfhydryl A chain which on recombination with B chain generates practically the same amount of insulin activity as when a 23-fold excess of thiol is used for the reduction step. Similarly, no difference in the final recombination yield was observed when the reaction time of the thiol with the S-sulfonate of the A chain was varied from 4 to 8 min. To eliminate as much as possible any mechanical losses in the process of securing the reduced A chain, extraction with ethyl acetate was used to remove the excess of mercaptoethanol after termination of the reduction step, omitting completely isoelectric precipitation and washings with aqueous buffer solutions. The sulfhydryl form of the A chain thus obtained was mixed with the S-sulfonate of the B chain and allowed to react in dilute solution (*ca.* 2.5 mg of protein/ml) of glycine buffer, pH 10.6 at 2°C for 18 to 22 hr. In 20 experiments the yield of insulin formed ranged from 60 to 80 per cent of theory. This yield is calculated on the basis of the S-sulfonate of the B chain used (uncorrected for water content of the chains which amounts to 5% per chain). When the recombination reaction is carried out by the same method but with large excess of B chain S-sulfonate, the yield of the insulin formed is only 17–21 per cent of theory, as calculated on the basis of the S-sulfonate of the A chain used prior to its conversion to the sulfhydryl form. From the recombination mixture of natural bovine insulin chains, crystalline insulin (Fig. 3) with a specific activity of 24 units was obtained with the method of isolation described by Smith.

We have recently used this novel method for combining our synthetic sheep insulin chains with the respective natural chains which have been prepared by oxidative sulfitolysis of bovine insulin. When the sulfhydryl form of the synthetic sheep insulin A chain^{5, 20} was recombined with the S-sulfonate form of the natural B chain of bovine insulin, the respective hybrid insulin was generated in yields ranging from 25 to 33 per cent (based on the B chain S-sulfonate used). Combination of the reduced, natural bovine insulin A chain with the synthetic S-sulfonate form of sheep insulin B chain⁶ led to the formation of the respective hybrid insulin in yields ranging from 10 to 13 per cent of theory (based on the B chain S-sulfonate used). Both hybrid (the one chain synthetic and the other chain natural) insulins have been isolated in crystalline form.^{9, 21} The reason the synthetic chains have a lower efficiency in generating insulin than have the chains prepared by oxidative sulfitolysis of natural insulin is not as yet clear. However, it might be pointed out that in the preparation of the sulfonates of the synthetic insulin chains the final step is treatment of the intermediate protected chains with sodium in liquid ammonia followed by oxidative sulfitolysis.^{5–8} When the S-sulfonates of the natural chains were prepared by sodium in liquid ammonia cleavage of insulin followed by oxidative sulfitolysis, they also exhibited a lower efficiency in generating insulin upon recombination. Specifically, when the S-sulfonate of the B chain had been prepared by the sodium in liquid ammonia route, the recombination yield was only 8 per cent; when the S-sulfonate of the A chain had been prepared by the sodium in liquid ammonia route, the recombination yield was 10 per cent. The nature of this phenomenon is now under investigation in our laboratory.

Summary.—A procedure is described by which natural chains prepared by oxidative sulfitolysis of bovine insulin are recombined to produce insulin in yields ranging from 60 to 80 per cent of theory. This procedure has been applied with favorable results to the combination of the synthetic chains of sheep insulin with the corre-

sponding chains of bovine insulin. The high recombination yields strongly suggest that as far as insulin is concerned, the essential information for the proper alignment of the insulin chains to produce the protein is contained within the primary structure of the chains.

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¹ Katsoyannis, P. G., *Metabolism*, **13**, 1059 (1964).

² Katsoyannis, P. G., *J. Polymer Sci.*, **49**, 51 (1961).

³ Dixon, G. H., and A. C. Wardlaw, *Nature*, **188**, 721 (1960).

⁴ Du, Y.-C., Y.-S. Zhang, Z.-X. Lu, and C.-L. Tsou, *Sci. Sinica (Peking)*, **10**, 84 (1961).

⁵ Katsoyannis, P. G., A. Tometsko, and K. Fukuda, *J. Am. Chem. Soc.*, **85**, 2863 (1963).

⁶ Katsoyannis, P. G., K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *J. Am. Chem. Soc.*, **86**, 930 (1964).

⁷ Katsoyannis, P. G., A. Tometsko, J. Ginos, and M. Tilak, *J. Am. Chem. Soc.*, **88**, 164 (1966).

⁸ Katsoyannis, P. G., A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, **88**, 166 (1966).

⁹ Katsoyannis, P. G., in "Brookhaven National Laboratory Symposium on Structure and Function of Polypeptide Hormones: Insulin," *Am. J. Med.*, in press.

¹⁰ Du, Y.-C., R.-Q. Jiang, and C.-L. Tsou, *Sci. Sinica (Peking)*, **14**, 229 (1965).

¹¹ Katsoyannis, P. G., and A. Tometsko, unpublished data.

¹² Smith, L. F., *Biochim. Biophys. Acta*, **82**, 231 (1964).

¹³ Zahn, H., B. Gutte, E. F. Pfeiffer, and J. Ammon, *Liebigs Ann. Chem.*, **691**, 225 (1966) state that oxidation of the reduced A chain with air at pH 8.8 for 60–100 min prior to its combination with reduced B chain could increase the yield of insulin formation to an average 20–30 per cent of theory. However, in this case too, the yield is not calculated on the basis of the amounts of the starting chains but on the basis of the specific activity of the final oxidation product.

¹⁴ du Vigneaud, V., C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).

¹⁵ du Vigneaud, V., D. T. Gish, P. G. Katsoyannis, and G. P. Hess, *J. Am. Chem. Soc.*, **80**, 3355 (1958).

¹⁶ Bodanszky, M., and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

¹⁷ Rydon, H. N., in *CIBA Foundation Symposium on Amino Acids and Peptides with Anti-metabolic Activity*, ed. G. E. W. Wolstenholme and C. M. O'Connor (London: J. & A. Churchill, Ltd., 1958), p. 192.

¹⁸ Swan, J. M., *Nature*, **180**, 643 (1957).

¹⁹ Humbel, R. E., these PROCEEDINGS, **53**, 853 (1965).

²⁰ Katsoyannis, P. G., A. Tometsko, and C. Zalut, to be published.

²¹ Katsoyannis, P. G., A. Trakatellis, A. Tometsko, S. Johnson, and G. Schwartz, unpublished data.